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ORIGINAL ARTICLE

WUS and *STM* homologs are linked to the expression of lateral dominance in the acaulescent *Streptocarpus rexii* (Gesneriaceae)

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Abstract Acaulescent species of *Streptocarpus* Lindl. show unusual patterns of growth, characterized by anisocotyly (i.e. the unequal growth of cotyledons after germination) and lack of a conventional embryonic shoot apical meristem (SAM). A SAM-like structure appears during post-embryonic development on the axis of the continuously growing cotyledon. Since we have shown previously that *KNOX* genes are involved in this unusual morphology of *Streptocarpus rexii*, here we investigated the expression pattern of *WUSCHEL* (*WUS*), which is also required for the indeterminacy of the SAM, but is expressed independently from *KNOX* in *Arabidopsis thaliana*. In *A. thaliana WUSCHEL* is involved in the maintenance of the stem cell fate in the organizing centre. The expression pattern of the *WUS* ortholog in *S. rexii* (*SrWUS)* strongly deviates from that of the model plant, suggesting a fundamentally different spatial and temporal regulation of signalling involved in meristem initiation and maintenance. In *S. rexii*, exogenous application of growth regulators, i.e. gibberellin (GA_3), cytokinin (CK) and a gibberellin biosynthesis inhibitor (PAC), prevents anisocotyly and relocates meristematic cells to a position of conventional SAMs; this coincides with a re-localization of the two main pathways controlling meristem formation, the *SrWUS* and the KNOX pathways. Our results suggest that the establishment of a hormone imbalance in the seedlings is the basis of anisocotyly, causing a lateral dominance of the macrocotyledon over the microcotyledon. The peculiar morphogenetic program in *S. rexii* is linked to this delicate hormone balance and is the result of crosstalk between endogenous hormones and regulatory genes.

Keywords Anisocotyly · Hormones · Lateral dominance · Shoot apical meristem · *Streptocarpus* · *SrWUS* · *SrSTM1*

Abbreviations

BAP	6-Benzylaminopurine
BM	Basal Meristem
CK	Cytokinin
CLV	*CLAVATA*
DAS	Days after sowing
DIG	Digoxygenin
FAA	Formaldehyde, acetic acid, ethanol
GA	Gibberellic acid
GM	Groove meristem
KNOX	*Knotted*-like homeobox genes
PM	Petiolode meristem
PAC	Paclobutrazol
RACE	Rapid amplification of cDNA ends
RAM	Root apical meristem
RT-PCR	Reverse transcriptase PCR
SAM	Shoot apical meristem
SrSTM1	*Streptocarpus rexii SHOOTMERISTEMLESS1* homolog
STM	*SHOOTMERISTEMLESS*

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SrWUS *Streptocarpus rexii WUSCHEL* homolog
WUS *WUSCHEL*

Introduction

Organ generation in plants is an indeterminate process depending on the activity of meristems. The shoot apical meristem (SAM) is located at the shoot apex and leaves, stems and axillary meristems are produced from its derivative cells. As the SAM controls the fate and positions of organs, it is the determinant for establishing the architecture of a plant (Carles and Fletcher 2003).

Acaulescent species of the genus *Streptocarpus* (Gesneriaceae), such as *S. rexii,* lack a conventional SAM and show unusual patterns of growth, characterized by a phyllomorphic structure (Jong and Burtt 1975; Mantegazza et al. 2007; Nishii and Nagata 2007). Linked to this phenomenon is anisocotyly, i.e., the unequal growth of cotyledons after germination. The development of the phyllomorph, a specific term introduced for leaves in *Streptocarpus* (Jong and Burtt 1975), is governed by three meristems in *S. rexii*: the basal meristem (BM) and the petiolode meristem (PM), both located at the proximal end of the lamina and responsible for the expansion growth of the phyllomorph, and the groove meristem (GM) positioned on the petiolode near the base of the lamina, from which further phyllomorphs are produced (Jong and Burtt 1975; Mantegazza et al. 2007; Nishii and Nagata 2007). Previous studies showed how the morphology of *Streptocarpus* species can be modulated experimentally by exogenous application of hormones (Rosenblum and Basile 1984; Nishii and Nagata 2007). In particular, the application of cytokinins (CK) to the acaulescent species *S. rexii* was found capable to promote isocotyly and a re-establishment of a central post-embryonic SAM (Mantegazza et al. 2007).

The role of phytohormones in controlling plant architecture has been largely investigated in model plants. Cytokinins are a class of plant hormones that promotes cell division and meristem function (Miller et al. 1955; Rupp et al. 1999; Werner et al. 2001). Exogenous cytokinin application upregulates the expression of CycD3, a cyclin that plays a key role in the regulation of plant cell division (Riou-Khamlichi et al. 1999). Experiments lowering the endogenous CK levels showed a reduced meristem size and leaf initiation rate in *Arabidopsis* (Werner et al. 2003), revealing the CK requirement for proper meristem activity. Recently, the direct regulation of meristem activity by the CK-activating enzyme *LONELY GUY* (*LOG*) demonstrated the positive role of CK in meristem function (Kurakawa et al. 2007). Another class of plant hormones, the gibberellins, affects the important aspects of plant growth including seed germination, cell elongation, and leaf initiation (Fleet and Sun 2005). It has been shown that the spatial restriction of its accumulation is crucial for the establishment of boundaries between meristem and leaf primordia (Jasinski et al. 2005).

Recent work has indicated complex interactions between hormones and transcription factors in controlling plant architecture. In particular, the homeobox genes *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*), encoding two major regulators of meristem formation and maintenance in *Arabidopsis*, have been shown to interact with phytohormones in determining meristem and organ formation. Several studies have provided evidence of positive regulation of CK by KNOX proteins. In fact, the ectopic activation of *Arabidopsis* KNOX proteins, including *STM*, in transgenic plants resulted in a rapid increase in the expression of the CK biosynthesis gene *ISOPENTENYL TRANSFERASE7* (*IPT7*) (Jasinski et al. 2005). Moreover, exogenous application of cytokinins partially rescued meristem defects of *stm* mutants, suggesting that KNOX action on meristem function is in part mediated by cytokinins (Yanai et al. 2005). *STM* has also been shown to positively regulate the expression of a gene encoding a GA_2-oxidase, which deactivates bioactive GAs. GA_2-oxidase expression in the boundary between the meristem and leaf primordium restricted the GA accumulation to the organ primordia (Jasinski et al. 2005). *KNOX* proteins also repress GA_{20}-oxidase gene expression in the SAM and hence GA biosynthesis, thus promoting meristem activity (Sakamoto et al. 2001; Jasinski et al. 2005). In addition, it has been reported that exogenous application of GA suppresses *KNOX* misexpression phenotypes in *Arabidopsis* and tobacco (Tanaka-Ueguchi et al. 1998; Hay et al. 2002).

Furthermore, a direct interaction between the *CLAVATA*/*WUSCHEL* (*CLV*/*WUS*) network and CK signalling has recently been shown to be required for proper meristem function (Leibfried et al. 2005). Thus, a combination of increased CK and reduced GA in the meristem mediated by *KNOX* and *CLV*/*WUS* activity has been indicated as crucial in meristem maintenance and organ production.

Previous analyses on the acaulescent species *S. rexii* have shown that the expression pattern of *SrSTM*, the ortholog of *STM*, strongly deviated from that of model plants (Mantegazza et al. 2007). Gallois et al. (2002) indicated that *STM* alone is insufficient for SAM formation, and other genes are required for meristem initiation and maintenance, e.g. *WUS*, which is expressed independently of *STM*. Thus, to further investigate processes involved in the determination of the peculiar morphology in acaulescent *Streptocarpus* species, we cloned and analyzed the expression of *SrWUS*, the ortholog of *WUS*, during *S. rexii* development.

Mantegazza et al. (2007) have indicated the existence of a cross talk between hormones and *KNOX* genes that could underlie anisocotyly and the unorthodox SAM formation in

this genus. However, the correlation of KNOX genes and GA is still to be tested particularly in the light of the hypothesis that uncoupling of the KNOX and GA pathway has occurred in *Streptocarpus* (Harrison et al. 2005). Moreover, the existence of a cross talk between the WUS/CLV pathway and hormones in acaulescent *Streptocarpus* species remains to be clarified. To shed light on the interaction between growth regulators and gene activities, *S. rexii* seedlings were treated with different plant growth regulators, i.e., 6-benzylaminopurine (BAP), gibberellin A_3 (GA_3), and the triazole type plant growth retardant paclobutrazol (PAC), a gibberellin biosynthesis inhibitor (Dai et al. 2007). In particular, we analyzed changes in morphology, and *SrWUS* and *SrSTM* expression induced by the hormone treatments. We will demonstrate that the hormones are able to change plant morphology, in synchrony with a delocalisation of *SrWUS* and *SrSTM* expression, in the acaulescent *S. rexii*.

Materials and methods

Plant materials

Plant and seed materials of *Streptocarpus rexii* Lindl. (Gesneriaceae; Lindley 1828), RBGE accession number 20030814 (Tsitsikamma, Cape Province, SA), came from the living research collection held at the Royal Botanic Garden, Edinburgh. Voucher specimens are deposited at Edinburgh (E).

Histological analysis

For the histological analysis of developing embryos, fruits were collected at weekly intervals from artificial pollinations until seed maturation. Stages of embryogenesis were identified according to the size, shape, and cell number present in each stage of development, as described by Mantegazza et al. (2007). For the analysis of primary phyllomorph development (*i.e.* macrocotyledon), seeds were sown on compost and collected every day until day 15 and then every week until the emergence of the first true non-cotyledonary phyllomorph, hereafter termed leaf for simplicity throughout the text.

Developing fruits and seedlings were fixed in FAA (formaldehyde 3.7% and acetic acid 5% in 50% ethanol) overnight at 4°C. After dehydration in an ethanol series (70–90–95–100%, for 1 h each), samples were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) and cut at 4-µm thickness. The sections were stained with DAPI (1 µg/ml for 5 min) and observed with a fluorescence microscope Dialux 22 (Leitz, Oberkochen, Germany).

For observation of the leaf vasculature, samples were fixed in FAA and decolourized overnight using ethanol. The decolourized tissue was hydrated in an ethanol series (95–90–70% for 1 h each) and then immersed in distilled water. Hydrated tissue was further cleared by immersion in an aqueous solution (consisting of chloral hydrate 200 g and glycerol 20 g in 50 ml of distilled water) for 3 h. Samples were observed with a stereomicroscope (Leica MZ6, Wetzlar, Germany).

Plant hormone treatments

Seeds were sterilized in 0.2% sodium hypochlorite for 2 min and washed with distilled water, then sown on a full strength MS basal medium (Murashige and Skoog 1962). Twelve days after sowing (DAS), just after cotyledon unfolding, seedlings were transferred to a medium containing 10 µM 6-benzylaminopurine (BAP), 10 µM gibberellic acid A_3 (GA_3), or 5 µM paclobutrazol (PAC). Control plants were grown on MS basal medium without growth regulators. For anatomical and in situ analysis, seedlings were collected at weekly intervals and fixed in FAA as described above.

Isolation of *SrWUS* sequences

RNA was extracted using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich, Saint Louis, MO, USA) and first strand cDNA was prepared using an oligo (dT) primer and 5 µg of total RNA and the Super Script kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

WUS-like sequences were amplified from cDNA using degenerated primers 'Fw' (5′-TACTACAACAATGGA/TGTTAGG-3′) and 'Re' (5′-TCCTCCTCTATCTCTAGGGTTT-3′), designed during the present study and located in conserved regions between *WUS* of *Arabidopsis thaliana* and *ROSULATA (ROA)*, the *WUS* ortholog in *Antirrhinum majus*. These primers were used in polymerase chain reaction (PCR) with 20 ng of cDNA and a thermocycler profile of 35 cycles of 94°C (50 s), 50°C (50 s) and 72°C (1 min). PCR products were purified with the GFX-PCR-DNA and Gel-Band-Purification kit (GE Healthcare, Buckinghamshire, UK) and either sequenced directly or after cloning using the pGEM-Teasy Vector System (Promega, Madison, WI, USA).

For 3′ RACE, reverse transcription from 1.5 µg of total DNAase-treated RNA was primed using the T17 adaptor (5′-ACTCGAGTCGACATCGATTTTTTTTTTTTTTT-3′) after Frohman (1995), with minor modifications. Nested PCR was performed using the 'RACE-primer' (5′-GACTCGAGTCGACATCG-3′) and primer 'WusSouF' (5′-GAGAGGCAGAAAAAGAGG-3′) designed during the

present study. A booster PCR using 1 µl of the PCR product was performed using the nested 'RACE-primer' and 'IPF1' (5′-CTGCCATGCAAATGCAGTAT-3′). For 5′ RACE, the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) was used following the manufacturer's instructions. Reverse transcription was primed with 'WPR' (5′-GATCAGCATTGATGTAGCAG-3′). PCR was then performed using the 'Abridged Anchor Primer' provided in the kit and the designed primer 'ISWRST' (5′-CATTGATGTAGCAGCTTTTC-3′). Finally, a booster PCR was performed on 1 µl of the 5′RACE PCR product, using the 'Abridged Universal Anchor Primer' and a newly designed primer 'WUSsouR' (5′-GAACCGA TGAAGATGGAAAACC-3′).

The full-length cDNA sequence of the *Streptocarpus WUS*-like gene was deduced by aligning the sequences of the 5′ and 3′ RACE products. The genomic sequence was obtained by PCR on genomic DNA using the cDNA flanking primers 'WUSfor1' (5′-TGACCCCACATTTCCA G-3′) and 'WUSrev1' (5′-GGCATACGTATCCATTAGC-3′) designed here. The product was purified with the GFX-PCR-DNA and Gel-Band-Purification kit (GE Healthcare), cloned using the pGEM-Teasy Vector System (Promega) and then sequenced. The *SrWUS* genomic sequence has been deposited in GenBank (accession number: EF490994).

SrWUS sequence analysis

WUS-related sequence data were obtained from GenBank (http://www.ncbi.nlm.nih.gov). The *SrWUS*, *WUS*, *TER* and *ROA* protein sequences were aligned using ClustalW 1.8 (Thompson et al. 1997) at http://www.ebi.ac.uk/clustalw. Alignment of the homeodomain coding sequences was performed using ClustalX 1.81 (Thompson et al. 1997). PHYLIP (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein 1989) was used on the coding sequence alignment to generate phylogenetic trees, using the "Distance" algorithm for distance calculation and performing one hundred bootstraps with "Neighbor-joining" algorithm to estimate the support of each branch. All accession numbers of the selected homeodomain sequences are listed in supplemental data (S1).

Southern blot analysis

Genomic DNA was extracted from the leaf tissue of *S. rexii* using the method of Chen and Dellaporta (1994). For Southern-blot hybridization, 5 µg of genomic DNA was digested with *Eco*RI, *Eco*RV, *Hin*dIII (Roche, Indianapolis, IN, USA). The resulting fragments were separated by electrophoresis, blotted onto positively charged membranes (Amersham) and hybridized following the manufacturer's instructions. Two different *SrWUS1*-specific probes were used, one corresponding to the full genomic sequence of *SrWUS*, a second corresponding to the probe used in in situ hybridization. They were obtained using the following primer combinations, respectively: 'WUSfor1' (5′-TGACCCCACATTTCC AG-3′) and 'WUSrev1' (5′-GGCATACGTATCCATTAG C-3′) and 'ISWF1' (5′-GTTAATTCGGGTGGTTTTCC-3′) and 'ISWRST' (5′-CATTGATGTAGCAGCTTTTC-3′). High stringency hybridization was performed in a Church and Gilbert (1984) buffer at 65°C.

RT-PCR

RNA was extracted using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich). First strand cDNA was prepared using an oligo (dT) primer and 5 µg of total RNA. The Super Script kit (Invitrogen) was used following the manufacturer's instructions. The primer pair 'ISWF1' and 'ISWRST' was used for specific amplification of *SrWUS*. RT-PCR with actin primers 5′-GCCAAAGCAGTGA TCTCTTTGCTC-3′ and 5′-CACTCCTGCCATGTATG TCGCTAT-3′ was used as standard.

In situ hybridization

Digoxigenin-labeled (DIG) *SrWUS* and *SrSTM1* RNAs were generated using an in vitro transcription kit (Roche Diagnostics, Mannheim, Germany). The DIG-antisense *SrWUS* probe was generated using primers 'ISWF1' (5′-G TTAATTCGGGTGGTTTTCC-3′) and 'ISWR1' (5′-TAA TACGACTCACTATAGGGCATTGATGTAGCAGCTT TTC-3′). We have also generated a sense probe with the primers 'senseWR1' (5′-GCATTGATGTAGCAGCTT TTC-3′) and 'senseWF1' (5′-TAATACGACTCACTATA GGGTTAATTCGGGTGGTTTTCC-3′) to evaluate the levels of background signal for the stages analysed. The *SrSTM1* probe was generated as described in Mantegazza et al. (2007).

Hybridization and immunological detection were performed as described in Lopez-Dee et al. (1999) with minor modifications. Hybridizations were carried out at 45°C overnight. The detection was performed using a Dig-detection kit (Roche Diagnostics). The anti-digoxigenin antibody was used at a 1:1,000 dilution.

Results

Isolation of *SrWUS*

To investigate the role of genes involved in meristem formation, we isolated cDNA sequences of *WUS* orthologs from *S. rexii.* The degenerated primers designed to exon

sequences, conserved between *WUS* and its orthologs in other species, amplified a 480-bp sequence, and the full-length cDNA was obtained by RACE.

Alignment of the predicted SrWUS protein to the putative orthologs in other species showed that SrWUS shares typical features of *WUS*-like genes, the homeodomain and the three conserved short sequence motifs at the C-terminal, an acidic domain, the *WUS* box (TLPLFPMH) and an EAR-like domain (ASLELTLN) (Fig. 1a) (Ohta et al. 2001; Stuurman et al. 2002; Haecker et al. 2004; Hiratsu et al. 2004; Kieffer et al. 2006). Conservation within the homeodomain of *WUS* and *SrWUS* was 82.6% at the amino acid level. A phylogenetic comparison of *SrWUS* with other *WUS*-like genes and *WUS* related homeobox genes (WOXs), using the highly conserved homeodomain nucleotide sequences, revealed the close evolutionary relationship of *SrWUS* with the *WUS*-like gene clade (Bootstrap = 92%) (Fig. 1b).

One *WUS*-like gene is present in *S. rexii*

Southern-blot analysis of *S. rexii* DNA performed with a labeled full-length DNA probe of *SrWUS* revealed one band when restricted with *Hin*dIII and *Eco*RV. Two bands were detected when the DNA was restricted with *Eco*RI, which is due to an internal restriction site for this enzyme (Fig. 1c). This banding pattern suggests the existence of only one *SrWUS* gene in the *S. rexii* genome.

SrWUS expression

In order to determine whether *SrWUS* expression may be associated with meristematic activity, we performed RT-PCR on different tissues of *S. rexii* plants. The cDNA fragments amplified by RT-PCR showed that *SrWUS* was expressed strongly in the petiolode (Fig. 1d). However, a weak expression was also detected in the proximal midvein region, fruit and flower. The levels of expression varied between samples but it is not certain whether these findings have biological significance since this experiment is not quantitative. The expression pattern of *SrWUS* during embryo and seedling development was further verified by RNA in situ hybridization (below).

SrWUS expression during embryo development

SrWUS mRNA was detected relatively uniform throughout the developing embryo (Fig. 2a–e). At the heart stage the expression was highest at the distal extremes of the embryo, in the cotyledons and at the putative site of the root apical meristem (RAM) (Fig. 2b). A similar pattern of expression was maintained throughout the transition from heart to torpedo stage and onwards till maturation (Fig. 2c–e). The sense probe showed no mRNA signal supporting the validity of our in situ patterns (Fig. 2f). *SrWUS* expression was never localized in the fork between the cotyledons (Fig 2d, e, arrow), the site of *WUS* expression in plants with a conventional embryonic SAM. A layered SAM was lacking in *S. rexii*. The specificity of the mRNA probe was verified by Southern blot analysis, which showed that it hybridized to only one DNA fragment in samples digested with different restriction enzymes (data not shown).

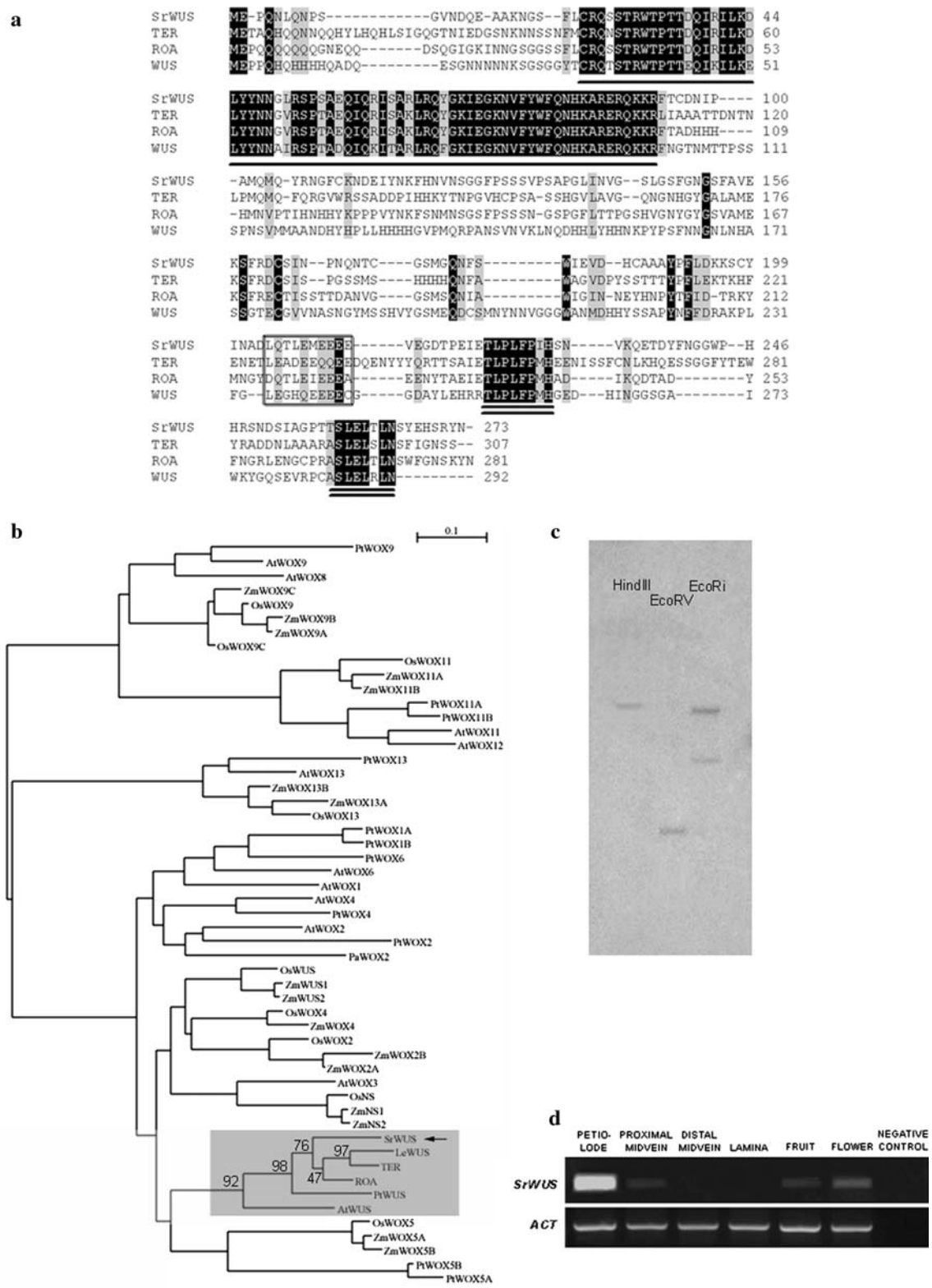
In situ hybridization during seedling development

To determine whether meristem initiation in *S. rexii* reflects initiation of the *WUSCHEL* pathway, localization of *SrWUS* transcripts was examined during germination and seedling growth. *SrWUS* expression was seen in root meristems at all stages (e.g., Fig. 3a). At 11 DAS *SrWUS* expression was localized throughout both cotyledons but was absent in between these (Fig. 3a). In seedlings 28 DAS, when anisocotyly becomes evident (Mantegazza et al. 2007), *SrWUS* was restricted to the base of the macrocotyledon in the BM (Fig. 3b). In 35 DAS, when the groove meristem arises on the petiolode (Mantegazza et al. 2007; Nishii and Nagata 2007), *SrWUS* expression was observed at the base of the expanding macrocotyledon and in the GM (Fig. 3c, arrow). During subsequent seedling development at the leaf primordial stage (Nishii and Nagata 2007), *SrWUS* expression was also detected in the basal part of the first leaf (Fig. 3d, arrow).

Hormone induced morphological variation

To investigate the role of hormones on the morphology of *S. rexii* seedlings, we histologically analysed the meristem initiation, and the development of cotyledons and vascular tissue in seedlings subjected to different hormonal treatments. Compared to untreated seedlings (Fig. 4a) the application of 6-benzylaminopurine (BAP) prevented the manifestation of anisocotyly (Fig. 4b), allowing meristems in the basal regions of both cotyledons to proliferate, resulting in the development of two macrocotyledons and isocotylous seedlings, possessing repositioned meristematic cells to a SAM like position (inset in Fig. 4b), as reported in Mantegazza et al. (2007). Our results further showed that the macrocotyledon of untreated seedlings (Fig. 4e, i) and both cotyledons of BAP-treated seedlings (Fig. 4f, j) possessed a complex branching vasculature, while the microcotyledon of untreated seedlings showed a simple, unbranched midvein (inset in Fig. 4i), as reported for other anisocotylous *Streptocarpus* species (Nishii et al. 2004).

The application of GA_3 also resulted in two equal-sized cotyledons (Fig. 4c), but with distinct petioles



(Fig. 4g) and microcotyledon features; i.e., small size and a simple unbranched midvein (Fig. 4g, k), suggesting the absence of basal meristem activity in both cotyledons. Similar to BAP, GA_3-treated seedlings showed a group of small meristematic cells located centrally between the cotyledons (Fig. 4m). Later on in seedling development, these cells gave rise to a leaf-like structure (Fig. 4o).

◀ **Fig. 1** Molecular characterization of *SrWUS*. **a** ClustalW alignment of *SrWUS*, *TER*, (*Petunia* × *hybrida WUS* AF481951) *ROA* (*Antirrhinum majus ROSULATA* AY162209) and *WUS* (*A. thaliana* AJ012310) proteins. Identical residues are highlighted in *black*, and similar residues are highlighted in *gray*. *Dashes* represent alignment gaps. The conserved acidic domain is *boxed*, and the homeodomain is *single underlined*. The two small conserved C-terminal (ASLELTLN) domains and *WUS* box (TLPLFPMH) are *double underlined*. **b** Neighbor-joining tree based on the WOXs homeodomains cDNA sequences in *S. rexii* and other plants (a complete list of samples is given in the supplemental data (S1)). The bootstrap values are shown only for the *WUS* clade, indicated in *gray*. *Arrow* shows *SrWUS*. **c** Southern analysis of *SrWUS*. Genomic DNA was digested as indicated. **d** Amplification of *SrWUS* by RT-PCR. Samples correspond to RNA extracted from six different tissues of *S. rexii* as indicated. Control amplifications of the constitutively expressed actin cDNA are shown below the *S. rexii* amplifications. *Negative control* amplification without cDNA

The application of PAC also induced the formation of two equal-sized cotyledons (Fig. 4d), but with a phenotype intermediate between GA_3- and BAP-treated seedlings, with some cotyledonary growth and occasionally branching midveins equally in both cotyledons (Fig. 4h, l). No dividing cells were found in the basal area of the cotyledon lamina at this point (Fig. 4n, p). A meristematic region was, however, located in the fork between the cotyledons (Fig. 4n), which subsequently developed into a leaf-like structure (Fig. 4p).

SrWUS and *SrSTM1* expression in hormone treated seedlings

To investigate in detail the expression pattern of *SrWUS* and *SrSTM1*, and evaluate their potential implication in the morphology of *S. rexii*, we performed in situ hybridization experiments on hormone-treated seedlings. In the early process of seedling development, we recognized three main morphological stages that were termed stages 1, 2 and 3, corresponding to the isocotylous, bulge and leaf formation stage described by Nishii and Nagata (2007). At stage 1 (Fig. 5a), just after cotyledon unfolding, the seedlings are isocotylous and have a concave region of small cells between the cotyledons, during stage 2 (Fig. 5b), a convex bulge of meristematic cells has formed, and at stage 3 (Fig. 5c), a leaf primordium has formed and the first leaf starts to differentiate.

In BAP-treated seedlings at stages 1 and 2, *SrWUS* transcripts were detected in the meristematic region between the cotyledons as well as in the BM of both macrocotyledons (Fig. 5d, e). Later, in the development of the seedlings, at stage 3, we observed a progressive re-localization of the signal from the central meristematic region toward groups of dividing cells in the first leaf-like structure. Moreover, the signal in the BM of enlarging macrocotyledons was still present (Fig. 5f).

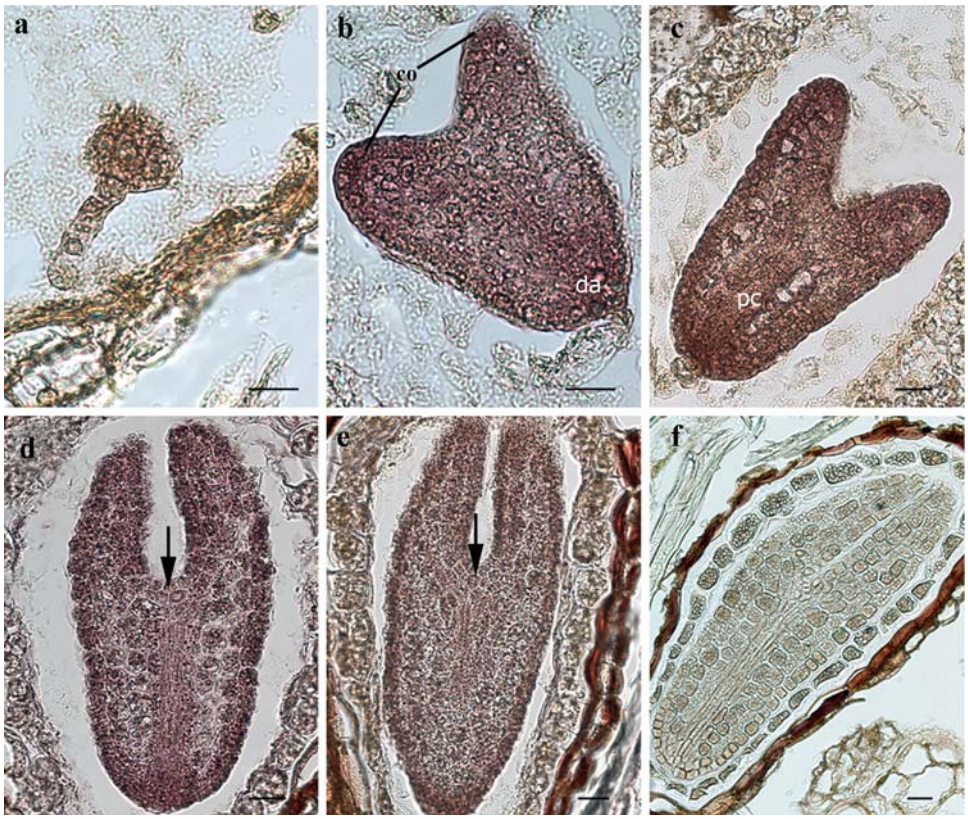


Fig. 2 *SrWUS* expression during different stages of embryogenesis in *Streptocarpus rexii*. *SrWUS* messenger hybridization is indicated by red–purple staining. **a** Globular stage. *SrWUS* is expressed throughout the embryo. **b** Early heart stage. A higher *SrWUS* expression is detected at the tip of both cotyledons (*co*) and the distal apex (*da*). **c** Transition stage. Additional in situ signal is present in the procambium (*pc*). **d–e** Linear cotyledon stage. *SrWUS* is not localized in the fork between the cotyledons. **f** Sense probe control on *S. rexii* embryo. In situ hybridization with the sense probe on the torpedo stages did not label any tissues. *Arrows* indicate where *SrWUS* like genes are usually detected in plants with a conventional SAM. Scale bars 20 μm (**a–f**)

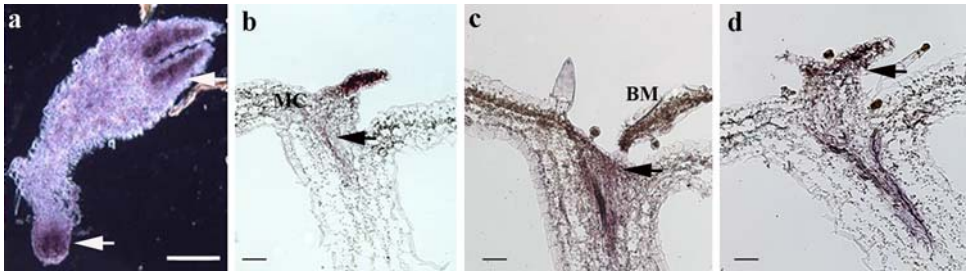


Fig. 3 *SrWUS* expression during seedling development in *Streptocarpus rexii*. *SrWUS* messenger hybridization is indicated by red-purple staining. **a** Eleven DAS. *Arrows* indicate *SrWUS* expression in both cotyledons and the root tip meristem. **b** 28 DAS. *SrWUS* expression is located at the base of the macrocotyledon (*MC*) in basal meristem (*BM*), and also in the vasculature (*arrow*). **c** 35 DAS. *Arrow* indicate *SrWUS* expression in the emerging groove meristem. Expression is also visible in the basal meristem (*BM*). **d** Longitudinal section through the first leaf. *Arrow* indicate *SrWUS* expression at the proximal region of the emerging leaf. Scale bars 100 μm (**a**–**d**)

In GA_3-treated seedlings at stage 1, the *SrWUS* signal was absent in the central concave region (Fig. 5g). Signals became evident at stage 2, in a small number of meristematic cells between the cotyledons (Fig. 5h, arrow) and at the tip of the leaf-like structure at stage 3 (Fig. 5i, arrow). No signals were detected in the basal region of cotyledons, where only large differentiated cells were evident histologically (Fig. 5g, h).

In PAC-treated seedlings, *SrWUS* mRNA messengers were first detected in central meristematic cells at stage 2 (Fig. 5j, arrow) and in dividing cells of the leaf-like structure at stage 3 (Fig. 5k arrow), respectively. *SrWUS* signals were absent in the basal region of the lamina of both cotyledons. *SrWUS* expression was detected in vascular tissue at all stages of development and in all treatments. *SrSTM1* expression in GA_3-treated seedlings was not detected until a cluster of meristematic cells became visible at stage 2 (Fig. 5m, arrow). At this stage, a weak signal was evident in a few meristematic cells in the central region between the cotyledons. At stage 3 (Fig. 5n), the hybridization signal became localized in the tip of the leaf-like structure arising from the central meristematic region. No signal was detected in the large cells at the base of the cotyledons (Fig. 5m).

In PAC-treated seedlings at stage 2, the expression of *SrSTM1* was observed in the bulge of meristematic cells (Fig. 5o, arrow). At stage 3, a strong signal was detected in the leaf-like structure emerging from between the two cotyledons (Fig. 5p). As in GA-treated seedling, no *SrSTM* signal was detected at the base of the cotyledons where only large cells were present (Fig. 5o, p).

Discussion

SrWUS, the *WUS*-like gene ortholog in *S. rexii*

We obtained *SrWUS* gene sequences from *S. rexii* that shared the same conserved motifs of other *WUS*-like genes, i.e., the homeodomain and the three conserved short-sequence motifs at the C-terminal (Stuurman et al. 2002; Haecker et al. 2004; Kieffer et al. 2006). The amino acid sequence in the homeodomain of *SrWUS* was also greatly conserved compared to *WUS*. These results, together with the phylogenetic analysis, strongly supports the orthology of *SrWUS* and *A. thaliana WUS*.

Delocalization of *SrWUS* and *SrSTM1* expression during embryogenesis reflects the absence of a SAM in *S. rexii*

In *A. thaliana WUS* is required to maintain a pool of stem cells in the SAM and it is expressed from the early phases of embryo development in a small group of cells in the central zone which will form the SAM organizing centre. In the embryo of *S. rexii*, where a SAM is not established, this pattern of *WUS* expression is not observed. In fact, *SrWUS* is expressed throughout the embryo, but never localized in the conventional position of a SAM, i.e. the fork between cotyledons. A similar pattern of expression in *S. rexii* has been shown for *SrSTM1* (Mantegazza et al. 2007), the homolog of *A. thaliana STM* that plays a crucial role in meristem formation and maintenance, preventing premature cell differentiation in the SAM (Endrizzi et al. 1996).

The peculiar expression patterns of *SrWUS* and *SrSTM1* during embryogenesis in *S. rexii* suggest that they are not sufficient to initiate and develop a layered embryonic SAM. The failure to establish such a SAM in *S. rexii* is not due to the absence of these genes. Since their transcription is maintained in *S. rexii*, the developmental significance of their expression is simply different from the model plant *Arabidopsis*. This is in line with previous observations showing how changes in the expression domain of main regulatory genes can underlie the evolution of morphological variation in plants (Hay and Tsiantis 2006).

SrWUS, *SrSTM1* and meristematic activity in *Streptocarpus*

In *A. thaliana*, the continuous organ formation from the SAM during the postembryonic phase depends on the

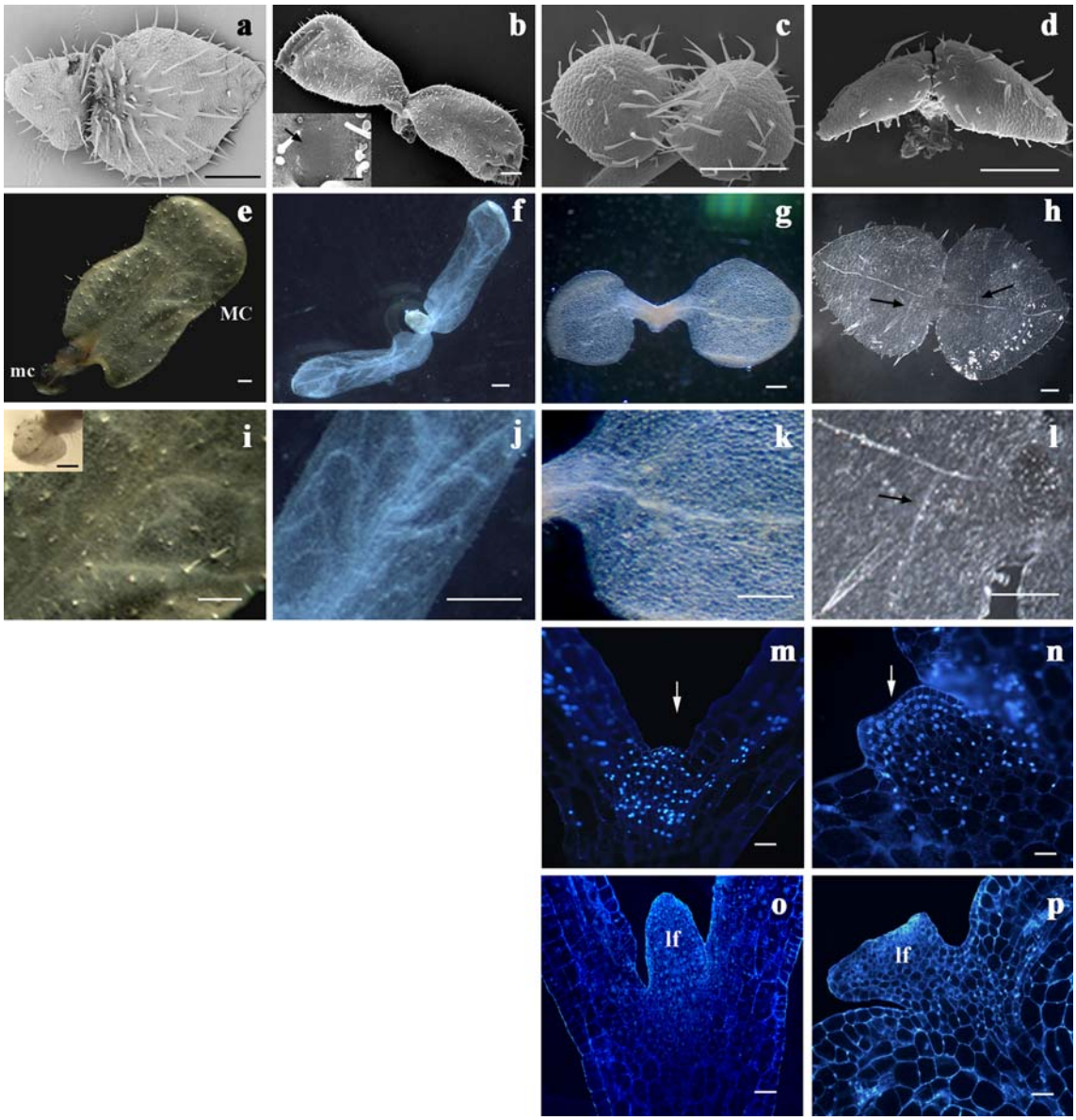
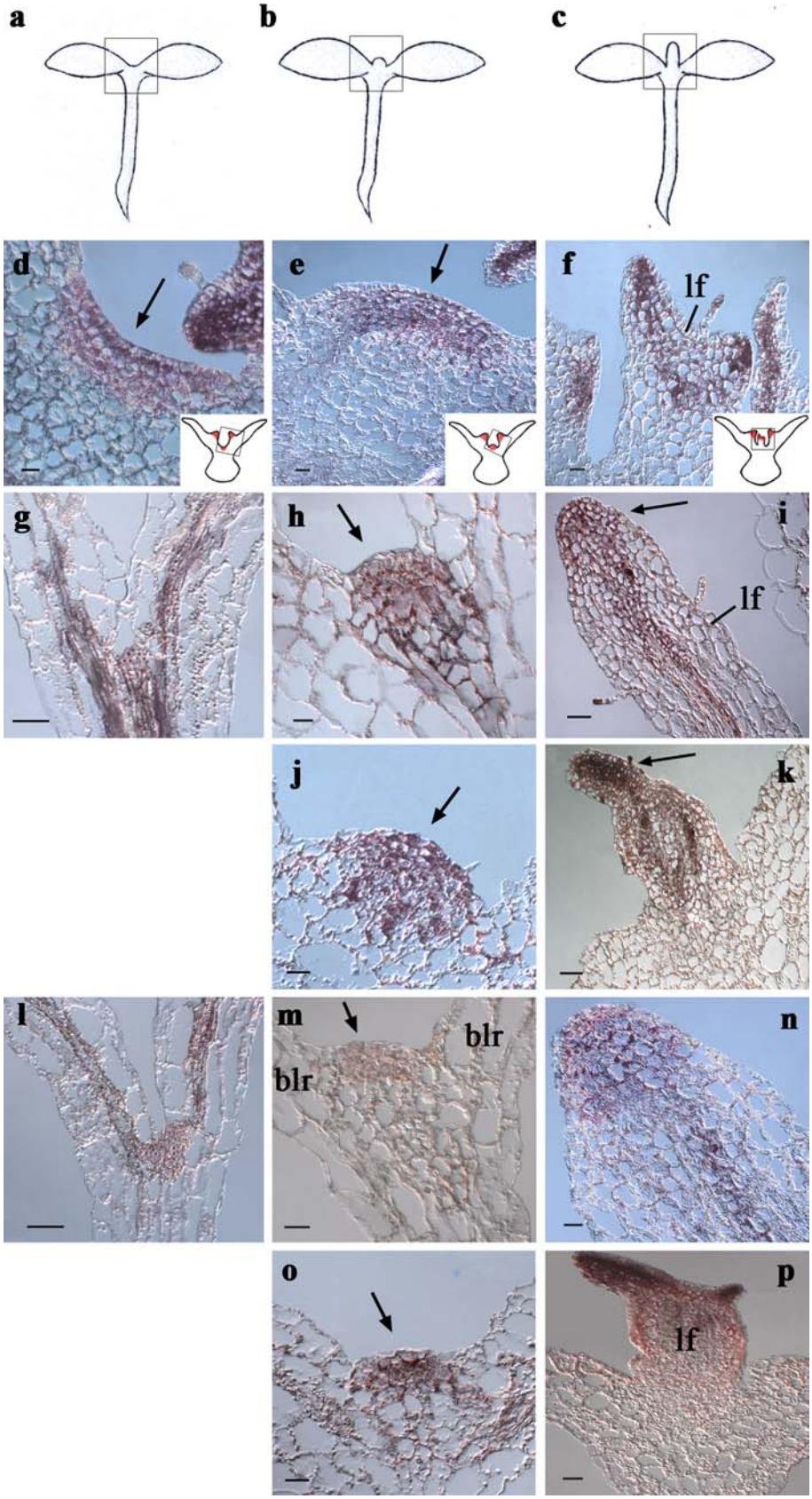


Fig. 4 Morphological analysis of hormone-treated seedlings of *Streptocarpus rexii*. **a–d** Scanning electronic micrographs showing isocotyly in hormone-treated seedlings. **a** Control seedling sown on MS medium without hormones. **b** BAP-treated seedling with two macrocotyledons. **c** GA_3-treated seedling with two microcotyledons. **d** PAC-treated seedling showing intermediate-sized cotyledons. **e–l** Cleared hormone-treated seedlings showing vascular tissue. **e**, **i** Control seedling sown on MS medium without hormones. **i** Magnification of (**e**) showing the complex vascular tissue in the macrocotyledon (*MC*). In the microcotyledon (*mc*) a simple midvein is present (*inset* in **i**). **f**, **j** BAP-treated seedling showing a complex vasculature. **j** Magnification of **f**. **g**, **k** GA_3-treated seedling with unbranched midveins. **k** Magnification of **g**. **h**, **l** PAC-treated seedling showing a simple midvein and occasional lateral vein (*arrows*). **l** Magnification of **h**. **m**, **o** DAPI stained longitudinal sections of GA_3 treated seedlings in two differents developmental stages. A limited number of small meristematic cells is evident in the central region (*arrow*) (**m**), which subsequently give rise to a leaf-like structure (*lf*) (**o**). **n**, **o** DAPI stained longitudinal sections of PAC-treated seedlings in two different developmental stages. **n** *Arrow* indicate the central meristematic region in the fork between two cotyledons. **p** A leaf-like (*lf*) structure emerging from the central meristematic region. *Scale bars* 0.6 mm (**e–l**), 500 μm (**a–d**), 100 μm (*inset* in **b**), 50 μm (**m–p**)

maintenance of stem cells in an undifferentiated state, which is achieved by the co-activity of *STM* and *WUS* (Endrizzi et al. 1996). Moreover, an ectopic co-expression of *WUS* and *STM* in *A. thaliana* has been shown to activate meristem functions, i.e. cell division, expression of meristem regulators, and organogenesis (Gallois et al. 2002). This suggests that both genes synergistically confer meristematic cell identity (Lenhard et al. 2002).

In acaulescent *Streptocarpus* species, such as *S. rexii*, a SAM is not established during embryogenesis and the development of new organs is due to the interplay of three meristematic regions at the junction of the lamina and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◀ **Fig. 5** *SrWUS* and *SrSTM1* expression in hormone-treated seedlings. **a–c** Drawings depicting the developmental stages 1, 2, and 3: stage 1 (**a**), stage 2 (**b**), and stage 3 (**c**); the *squares* indicate the areas shows in the photos. **d–k** In situ hybridization with a *SrWUS* probe on hormone-treated seedlings at different developmental stages. **d–f** BAP-treated seedlings. *Arrows* indicate *SrWUS* expression in a central meristem at stage 1 (**d**) and 2 (**e**). **f** *SrWUS* signal localization in several foci of dividing cells in the leaf-like (*lf*) structure. Strong signals are also observed in the basal meristem (*BM*) of both cotyledons in all stages analyzed. *Insets* in **d, e,** and **f** are schematic illustrations of BAP-treated seedlings showing *SrWUS* expression (labeled in *red*) at the stages 1, 2 and 3, respectively; the *squares* indicate the area depicted in the micrographs. **g–i** GA_3-treated seedlings. At stage 1 (**g**), no *SrWUS* signal is detected between the cotyledons. At stage 2 (**h**) *SrWUS* transcripts are evident in a small number of meristematic cells between the cotyledons (*arrow*). **i** *SrWUS* expression on the tip of the leaf-like (*lf*) structure emerging from the central meristematic region. **j–k** PAC-treated seedlings. At stage 2 (**j**), *SrWUS* expression is located in the central meristematic cells (*arrow*), at stage 3 (**k**), in dividing cells in the distal region of the leaf-like (*lf*) structure. **l–p** In situ hybridization with a *SrSTM1* probe on seedlings treated with hormones. **l–n** GA_3-treated seedlings. **l** No *SrSTM1* signal is detected in stage 1 seedlings. **m** Stage 2. *Arrow* indicates *SrSTM1* expression in few meristematic cells in the central convex region. **n** At stage 3, the hybridization signal is localized at the tip of the leaf-like (*lf*) structure. No signal is detected in cells corresponding to the basal lamina region (*blr*) (**m**). **o–p** PAC-treated seedlings. **o** Expression of *SrSTM1* is restricted to the bulge of meristematic cells at stage 2 (*arrow*). **p** Only the leaf-like (*lf*) structure shows *SrSTM1* transcripts. *SrWUS* and *SrSTM1* signals are also detected in the vascular tissue of all seedlings. *Scale bars* 20 μm (**e**, **j, k, n**, **o**), 50 μm (**d**, **f**, **g–i**, **l**, **m**, **p**)

petiolode during post-embryonic development (Jong 1970). In all three of these regions both *SrWUS* and *SrSTM1* are co-expressed, suggesting a conserved role of these genes in inducing cell division and suppressing cell differentiation, associated with a novel molecular mechanism that may be involved for the unusual morphology in *S. rexii*.

Recent findings indicate that the regulation of *WUS* transcription is a central checkpoint in the control of stem cells in the SAM in *A. thaliana*. Particularly, the size of the stem cell population is controlled through the *WUS* expression domain, achieved through integrating information from several regulatory pathways (Bäurle and Laux 2005). In *S. rexii*, the *SrWUS* domain is much broader throughout the meristematic cells, even extending into the BM in cotyledons, suggesting a coupled delocalization of the main spatial regulators of *WUS* expression, i.e., the feedback loop *WUS*/*CLV* pathway.

It is worth noting that *SrSTM* (Mantegazza et al. 2007) and *SrWUS* are expressed in both cotyledons until the onset of anisocotyly (Fig. 3). This is in line with findings by Nishii and Nagata (2007) of cell divisions in both cotyledons of *S. rexii* prior anisocotyly and with observations that for about 2 weeks after germination both cotyledons of *Monophyllaea*, another anisocotylous Gesneriaceae genus, retain the capability to form the macrocotyledon (Tsukaya 1997).

Anisocotyly inhibition relocates meristematic cells in a conventional SAM position

The results of our growth regulator experiments revealed that the hormones are key factors during the early development in establishing the decisive morphology of *S. rexii* seedlings. In accordance with Rosenblum and Basile's (1984) observations, our results demonstrate that the exogenous hormone applications are able to change the morphogenetic pattern in *S. rexii*. In particular, changes in the endogenous hormone balance by direct GA_3 or CK administration, or by inhibiting gibberellin biosynthesis (via PAC) were able to interfere with the cotyledonary accrescence, inducing isocotyly (regardless of the size of the cotyledons; GA → microcotyledons, CK → macrocotyledons). The establishment of isocotyly, either way, resulted in a re-localization of the GM to the orthodox position of the SAM, suggesting that anisocotyly and dislocation of the SAM are in the same developmental pathway, i.e., linked to the proliferation of the macrocotyledonary petiole, the petiolode.

The hormone-induced morphological changes, i.e., isocotyly and central meristem, provide evidence that the genetic regulation underlying a conventional growth pattern is apparently retained in *S. rexii* and reemployed under exogenous hormonal treatments.

SrSTM1 and *SrWUS* expression mark meristematic activity

Several publications indicate *WUS* and *STM* as main factors of the identity and maintenance of meristematic regions (Endrizzi et al. 1996; Lenhard et al. 2002).

In *S. rexii*, these genes were co-expressed and marked regions of actively dividing cells during germination and seedling development. Interestingly, in hormone-treated seedlings, sites of *SrWUS* and *SrSTM* expression reflected positional shifts of meristematic regions. These data confirm that these genes show a conserved function in the formation of meristems also in *S. rexii* and on the other hand support the hypothesis of a different spatial and temporal regulation of the *SrWUS* and *SrSTM* co-expression domain compared to *A. thaliana*.

Link between hormones and morphology

The opposing roles for CK and GA for plant development have previously been documented, underlining the requirement of high level of CK and low level of GA for proper meristem function (Jasinski et al. 2005). The phenotypes of hormone-treated seedlings confirm this antagonistic role of CK and GA on meristem activity also for *S. rexii*; in fact both hormonal treatments induced isocotyly and central meristems through the BAP-mediated induction or the GA-mediated inhibition of cell division in the putative cotyledonary basal meristems.

Hormones and developmental genes

Recent data from *Arabidopsis* and other species (e.g. tobacco; Sakamoto et al. 2001) show that KNOXs proteins repress GA (Hay et al. 2002) and activate CK biosynthesis (Yanai et al. 2005). Additionally, it has been reported that CK activity is necessary and sufficient, for stimulating the expression of GA catabolic genes, thereby reinforcing the low GA regime established by KNOX proteins in the SAM (Jasinski et al. 2005). Moreover, also *WUS* has recently been shown to participate in increasing CK level in meristems, by repressing the transcription of several *ARABIDOPSIS RESPONSE REGULATORS* genes, which act in the negative feed back loop with CK (Kiba et al. 2003; To et al. 2004; Leibfried et al. 2005).

In *S. rexii*, different hormone treatments prevent anisocotyly, along with delocalization of *SrWUS* and *SrSTM* expression. The findings are critical for the interpretation of the developmental control of anisocotyly in acaulescent anisocotylous *Streptocarpus* species. They are consistent with a model that envisages a concentration gradient of hormones across cotyledons that could be established by differences in cotyledonary *KNOX* and *WUS* expression level.

In untreated seedlings, a high GA level in the microcotyledon and a high CK level in the macrocotyledon can be deduced. This hormone arrangement is responsible for anisocotyly setting, and may be established by differences in GA repression due to differences in the level of KNOX expression in the two cotyledons; by this model in macrocotyledons, *SrSTM* represses GA and activates CK biosynthesis that in turn promotes GA deactivation; in parallel, *SrWUS* expression regulates cytokinin-inducible response genes, creating an environment with high CK and low GA levels, supporting meristem function. Clues as to how 'lateral dominance' of the macrocotyledon over the microcotyledon is established comes from light exposure experiments. In those experiments, the cotyledon in a pair that receives more light will become the macrocotyledon (Saueregger and Weber 2005). In *Arabidopsis*, light profoundly influences hormone networks involving bioactive GA level (Nemhauser 2008). How light is ultimately translated into a peculiar phenotype in *S. rexii* through a hormone balance is yet unclear.

In exogenously applied hormone-treated seedlings, GA may impair the concentration gradient in the cotyledons and suppress the meristematic activity in both cotyledonary BMs, thus causing isocotyly. As a result, the GM arises between the cotyledons where both *SrSTM* and *SrWUS* expression occur. The displaced position of the GM, in place of ordinary SAMs, is due to the non-proliferation of the petiolode of the macrocotyledon, i.e. the symmetrical development of both cotyledons.

The suggestion that GA is a key factor in anisocotyly is supported by PAC morphology. In PAC treated seedling no GA synthesis occurs, thus no difference in GA concentration is established between cotyledon, this in turn suppresses lateral dominance and relocates GM to a central position.

In CK-treated seedlings, the exogenously applied hormone leads to the situation that endogenous CK concentrations are sufficiently high to abolish the establishment of a lateral dominance and a GA induced inhibition of microcotyledon growth. A possible explanation may be that CK directs the activation of *GA2ox* genes and a positive feed back loop regulation between *STM*-like genes and CK dependent processes (Hay et al. 2004).

In the present work, we have demonstrated that the unorthodox development of the acaulescent *Streptocarpus rexii*, with chronological separation of germination, establishment of lateral dominance of one cotyledon, followed by GM initiation and phyllomorph development, may involve a cross talk between an endogenous hormone balance and genes implicated in meristem formation and maintenance.

Since light seems to be the factor determining which cotyledon becomes dominant, a direct measurement of hormone concentrations in different tissues in responses to light exposure during seedling development would be the next logical step in order to better understand the genetic mechanisms underlying the establishment of acaulescence and anisocotyly in acaulescent *Streptocarpus* species.

This is an interesting area of research with the potential to yield novel insights into the balance between the conservation of gene interactions, and the rise of novel ones that may be a prerequisite for attaining unorthodox growth morphologies in plants, as certainly is the case in the genus *Streptocarpus*, as an adaptive response to changing environments.

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